PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : **C12Q 1/68**	**A2**	(11) International Publication Number: **WO 99/09210** (43) International Publication Date: 25 February 1999 (25.02.99)

<table>
<tr><td>

(21) International Application Number: PCT/EP98/05175

(22) International Filing Date: 14 August 1998 (14.08.98)

(30) Priority Data:
    055,863   15 August 1997 (15.08.97)   US

(71) Applicant *(for all designated States except US)*: INSTITUT PASTEUR [FR/FR]; 28, rue du Docteur Roux, F–75724 Paris Cedex 15 (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PETIT, Christine [FR/FR]; 2, rue Amélie, F–92350 Le Plessis–Robinson (FR). DENOYELLE–GRYSON, Françoise [FR/FR]; 22, rue du 8 mai 1945, F–94110 Arcueil (FR). WEIL, Dominique [FR/FR]; 11, rue de Javel, F–75015 Paris (FR). MARLIN–DUVERNOIS, Sandrine [FR/FR]; 18, avenue J. Binet, F–92000 Colombes (FR). GUESDON, Jean–Luc [FR/FR]; 33, Grande Rue, F–92310 Sèvres (FR).

(74) Agent: HIRSCH, Denise; Cabinet Lavoix, 2, place d'Estienne d'Orves, F–75441 Paris Cedex 09 (FR).

</td><td>

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
    Without international search report and to be republished upon receipt of that report.

</td></tr>
</table>

(54) Title: A MUTATION WITHIN THE CONNEXIN 26 GENE RESPONSIBLE FOR PRELINGUAL NON–SYNDROMIC DEAFNESS AND METHOD OF DETECTION

(57) Abstract

A purified polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which in a wild form encodes a polypeptide implicated in hereditary sensory defect, wherein said mutated purified polynucleotide presents a mutation responsible for prelingual non–syndromic deafness selected from the group consisting of a specific deletion of at least one nucleotide.

A MUTATION WITHIN THE CONNEXIN 26 GENE RESPONSIBLE FOR PRELINGUAL NON–SYNDROMIC DEAFNESS AND METHOD OF DETECTION

BACKGROUND OF THE INVENTION

The present invention concerns a mutation responsible

10   for autosomal prelingual non-syndromic deafness and a method

for the detection of this hereditary sensory defect for

homozygous and heterozygous individuals. The invention

concerns more particularly a specific deletion of at least

one nucleotide in the connexin 26 (Cx 26) gene and especially

15   in a guanosine rich region, notably between the nucleotides

27 and 32. The invention is also directed to the use of

polynucleotide, or fragments thereof, for example as tools

useful for the *in vitro* detection of a mutation of a gene

belonging to the Cx26 gene family.

20   Profound or severe prelingual deafness affects one child

in a thousand in developed countries (Morton NE. Genetic

epidemiology of hearing impairment. In *Genetics of hearing

impairment*. (The New York Acad Sci, New York 1991; **630**:16-

31). It is a major handicap as it impedes language

25   acquisition.

According to studies performed in a U.S. population of

children with non-syndromic (isolated) prelingual deafness

and in whom an obvious environmental cause has been excluded,

it is estimated that up to two-thirds of the cases have a genetic basis (Marazita ML, Ploughman LM, Rawlings B, Remington E, Arnos KS, Nance WE. Genetic epidemiological studies of early-onset deafness in the U.S. school-age

5   population. *Am J Med Genet* 1993; **46**:486-91). These forms are mainly sensorineural and are almost exclusively monogenic. The major mode of inheritance is autosomal recessive (DFNB), involving 72% to 85% of cases, this fraction increasing to 90% when only profound deafness is

10 taken into account.

Autosomal recessive prelingual deafness is known to be genetically highly heterogeneous. Estimates of the number of DFNB loci vary from thirty to one hundred (Petit C. Autosomal recessive non-syndromal hearing loss. In *Genetics and*

15 *Hearing Impairment.* Martini A, Read AP, Stephens D, eds (Whurr, London) 1996; 197-212), for a review), of which fourteen have so far been mapped to the human chromosomes (Petit C. Genes responsible for human hereditary deafness: *symphony of a thousand. Nature Genet* 1996; **14**:385-91) for

20 review, (Verhoeven K, Van Camp G, Govaerts PJ, et al. A gene for autosomal dominant non-syndromic hearing loss (DFNA12) maps to chromosome 11q22-24. *Am J Hum Genet* 1997; **60**:1168-74 and Campbell DA, McHale DP, Brown KA, et al. A new locus for non-syndromal autosomal recessive sensorineural hearing loss

(DFNB16) maps to human chromosome 15q21-q22. *J Med Genet* 1997; in press).

A majority of the families attending genetic counseling clinics consist of normal hearing parents with a single deaf ·

5   child who wish to know the risk of recurrence of the defect. In most cases, given the major role of environmental causes of prelingual deafness, it is not usually possible even to recognize whether the hearing loss is of genetic origin. Genetic counseling in such families would be greatly improved

10   by an ability to detect DFNB mutations. In this respect, the high genetic heterogeneity of the condition represents a major obstacle.

After the initial identification of the DFNB1 locus on 13q11 in a large consanguineous Tunisian family (Guilford P,

15   Ben Arab S, Blanchard S, et al. A non-syndromic form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. *Nature Genet* 1994; 6:24-8), two studies performed on·New Zealand/Australian families (Maw MA, Allen-Powell DR, Goodey RJ, et al. The contribution of the

20   DFNB1 locus to neurosensory deafness ·in a Caucasian population. *Am J Hum Genet* 1995; 57:629-35), and on Italian/Spanish families (Gasparini P, Estivill X, Volpini V, et al. Linkage of DFNB1 to non-syndromic neurosensory autosomal-recessive deafness in Mediterranean families. *Eur*

4

J Hum Genet 1997; 5:83-8) suggested that this locus might be a major contributor to prelingual deafness in these populations, although individual lod scores obtained in these families were not significant owing to the small size of

5   these families.

Recently, the Cx26 gene, which encodes a gap junction protein, connexin 26, has been shown to underlie DFNB1 deafness. Two different G—>A substitutions resulting in premature stop codons in three DFNB1 linked consanguineous

10   Pakistani families have been reported (Kelsell DP, Dunlop J, Stevens HP, et al. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* 1997; **387**:80-3). These two substitutions were identified, respectively, at codon 77 and at codon 24.   This result has offered the

15   opportunity directly to assess this hypothesis.

The difficulties encountered in genetic counseling for prelingual non-syndromic deafness due to the inability to distinguish genetic and non-genetic deafness in the families presenting a single deaf child was one of the reasons that

20   led the inventors to undertake a characterization of the spectrum and prevalence of mutations present in the Cx26 gene in 35 families from several parts of the world with autosomal recessive prelingual deafness.

5

SUMMARY OF THE INVENTION

The determination of a mutation in the Cx26 gene has notably rendered possible the use of a detection probe as a
5   tool for the identification of a specific form of autosomal prelingual non-syndromic deafness, and more particularly the useful role of a newly identified 30delG (a G deletion at position 30; position 1 being the first base of the initiator codon) mutation in such families. This invention establishes
10 that the contribution of the DFNB1 locus predominantly results essentially from the 30delG mutation.   It is now believed that the 30delG accounts for about three-quarters of all recessive DFNB1 mutations.

The invention is thus intended to provide a purified
15 polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which in a wild form encodes a polypeptide implicated in hereditary sensory defect. The mutated purified polynucleotide presents a mutation responsible for prelingual non-syndromic deafness.
20   The invention also provides oligonucleotides comprising of 15 to 50 consecutive nucleotides of the mutated purified polynucleotide that are useful as primers or as probes.

In addition, the invention aims to supply a method and a kit for the detection of the hereditary sensory defect for
25 homozygous as heterozygous individuals.

According to the invention, the purified polynucleotide having a chain of nucleotides corresponding to a mutated sequence, which encodes in a wild form a polypeptide implicated in hereditary sensory defect, presents a mutation

5   responsible for prelingual non-syndromic deafness selected from the group consisting of a specific deletion of at least one nucleotide.

By mutation, according to the invention it means a specific deletion of at least one nucleotide. Thus, a

10   mutated sequence means a polynucleotide sequence comprising at least a mutation.

A chain of nucleotides, according to the invention, means a polynucleotide, which encodes not necessarily a polypeptide, but which presents between 27 and 2311

15   nucleotides linked together.

The invention particularly concerns a purified polynucleotide wherein, the specific mutation is a deletion located in a region encoding connexin 26 of chromosome 13q11-12, preferably located in a guanosine rich region starting at

20   nucleotide 27 preferably at nucleotide 30, and extending to nucleotide 32 or nucleotide 35, all the recited nucleotides being inclusive. More particularly according to the invention, the specific deleted purified polynucleotide encodes for a truncated polypeptide.

By truncated polypeptide, according to the invention it means a fragment of the polypeptide, which does not present the properties of the wild form of the polypeptide either in length, in amino acid composition, or in functional

5   properties.

A preferred embodiment of a specific deletion is a guanosine deletion at position 30, also called "30delG mutation". Another preferred embodiment of the specific deletion is a 38 bp deletion beginning at position 30.

10   The invention also includes a purified polynucleotide, which hybridizes specifically with any one of the polynucleotides as defined above under the following stringent conditions: at low temperatures between 23°C and 37°C, in the presence of 4 x SSC buffer, 5 x Denhardt's

15   solution, 0.05% SDS, and 100µg/ml of salmon sperm DNA. (1 x SSC corresponds to 0.15 M NaCl and 0.05M sodium citrate; 1 x Denhardt's solution corresponds to 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin).

The invention also concerns an oligonucleotide useful as

20   a primer or as a probe comprising 15 to 50 consecutive nucleotides of the polynucleotide according to any one of the polynucleotides as defined above. The oligonucleotide sequence is selected from the following group:

- A first couple:

8

5'-TCTTTTCCAGAGCAAACCGCC(SEQ ID No. 1)-3'

5'-TGAGCACGGGTTGCCTCATC(SEQ ID No. 2)-3'.

The length of the PCR product has been obtained from 285

bp in length;

5   - A second couple allowing to explore the other part of

the reading frame:

5'-GACACGAAGATCAGCTGCAG(SEQ ID No. 3)-3'

5'-CCAGGCTGCAAGAACGTGTG(SEQ ID No. 4)-3'

- A third couple:

10   5'-CTAGTGATTCCTGTGTTGTGTGC(SEQ ID No. 9)-3'; and
5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3' and
- A fourth couple:

5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTAGTGATTCCT
GTGTTGTGTGC(SEQ ID No. 14)-3'; and

15   5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3'.

Another oligonucleotide useful as a probe is selected

from the following group:

5'-AGACGATCCTGGGGGTGTGAACAAA(SEQ ID No. 5)-3'

20   5'-ATCCTGGGGGTGTGA(SEQ ID No. 6)-3'

5'-AGACGATCCTGGGGGCTCACCGTCCTC(SEQ ID No. 7)-3'.

In addition, the invention concerns a method for the

detection of an hereditary sensory defect, namely autosomal

prelingual   non-syndromic   deafness,   for   homozygous   as

25 heterozygous individuals in a biological sample containing

DNA, comprising the steps of:

a) bringing the biological sample into contact with a oligonucleotide primers as defined above, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization

5   of the primers with the DNA contained in the biological sample;

b) amplifying the DNA;

c) revealing the amplification products;

d) detecting the mutation.

10   Step d) of the above-described method may comprise a Single-Strand Conformation Polymorphism (SSCP), a Denaturing Gradient Gel Electrophoresis (DGGE) sequencing (Smith, L.M., Sanders, J.Z., Kaiser, R.J., Fluorescence detection in automated DNA sequence analysis. *Nature* 1986; **321**:674-9); a

15   molecular hybridization capture probe or a temperature gradient gel electrophoresis (TGGE).

Step c) of the above-described method may comprise the detection of the amplified products with an oligonucleotide probe as defined above.

20   According to the invention, a biological sample can be a blood sample extracted from people suffering from any kind of deafness with any criteria as follows: neurosensorial or mixed isolated deafness, advanced or not, at any degree of severity, concerning familial or sporadic case, or

individuals exposed to noise, or individuals suffering from a low acoustic, or individuals susceptible to carry an anomaly in the gene, or from an embryo for antenatal diagnostic.

5   Another aim of the invention comprises a method for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous and heterozygous individuals in a biological sample containing DNA, comprising the steps of:

10   a) bringing the biological sample into contact with an oligonucleotide probe according to the invention, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the
15   biological sample; and

b)   detecting the hybrid formed between the oligonucleotide probe and the DNA contained in the biological sample.

Step b) of the above-described method may consist in a
20   single-strand conformation. Polymorphism (SSCP), a denaturing gradient gel electrophoresis (DGGE) or amplification and sequencing.

The invention also includes a kit for the detection of an hereditary sensory defect, the autosomal prelingual non-

syndromic deafness, for homozygous as heterozygous

individuals, said kit comprising:

a) oligonucleotides according to the invention;

b) the reagents necessary for carrying out DNA

5   amplification; and

c) a component that makes it possible to determine the

length of the amplified fragments or to detect a mutation.

BRIEF DESCRIPTION OF THE DRAWINGS

10

This invention will be more described in greater detail

by reference to the drawings in which:

Figure 1 depicts the results of temperature gradient gel

electrophoresis for detection of mutants in which:

15   Lanes 1 and 2: DNA from normal patients.
          Lanes 3 and 4: DNA from homozygous patients with
                         30delG mutation.
          Lanes 5 and 6: DNA from heterozygous patients.
          Lane 7:   PCR control without DNA.
20   Lane 8:   PCR fragment amplified from a normal
                         DNA and hybridized with a standard DNA
                         fragment harboring the 30delG mutation.
          Lane 9:   PCR fragment amplified from a mutant
                         homozygous DNA and hybridized with a
25   normal standard DNA fragment harboring
                         the guanine 30.

DETAILED DESCRIPTION OF THE INVENTION

30

Prelingual non-syndromic (isolated) deafness is the most

frequent hereditary sensory defect in children.   The

inheritance in most is autosomal recessive. Several dozens of

genes might be involved, only two of which, DFNB1 and DFNB2,

have so far been identified (Kelsell, D.P., et al., Connexin
26 mutations in hereditary non-syndromic sensorineural
deafness. *Nature* 1997; **387**:80-3; Liu, X-Z, et al., Mutations
in the myosin VIIA gene cause non-syndromic recessive
deafness, *Nature Genet* 1997; **16**:188-90; and Weil, D., et al.,
The autosomal recessive isolated deafness, DFNB2, and the
Usher 1B syndrome are allelic defects of the myosin-VIIA.
Nature Genet 1997; **16**:191-3). A search was made searched for
mutations in the gene encoding connexin 26, Cx26, which has
recently been shown to be responsible for DFNB1. Mutation
analysis of Cx26 was performed by PCR amplification on
genomic DNA and sequencing of the single coding exon.

Example 1: Patients

Thirty-five affected families from various geographical
regions, mainly France, New Zealand and Australia, Tunisia
and Lebanon, were studied. They could be classified into
three categories: (1) consanguineous families each having a
significant linkage to the DFNB1 locus; (2) small non-
consanguineous families in which linkage analysis was
compatible with the involvement of DFNB1; and (3) small
families in which no linkage analysis had been undertaken.

The first category consists of six large families living
in geographically isolated regions. Five were from Tunisia,

13

two from the north and three from the south. Linkage to the

DFNB1 locus in the two families from northern Tunisia

(families 20 and 60) had previously been reported (Guilford

P, Ben Arab S, Blanchard S, et al., A non-syndromic form of

5   neurosensory, recessive deafness maps to the pericentromeric

region of chromosome 13q. *Nature Genet* 1994; 6:24-8); the

three families from southern Tunisia (S15, S19 and ST) and

the family from Lebanon (LH) comprise total of three, five,

two, and five deaf children, respectively, the deafness being

10   of severe or profound degree. The marriages were between

first cousins (S15, ST and LH) and between first and second

cousins (S19).   Linkage analysis of these six families

resulted in individual lod scores ranging from 2.5 to 10 with

polymorphic markers from the DFNB1 region (D13S175, D13S141,

15   D13S143 and D13S115).

The second category of patients comprises seven New

Zealand families with at least two deaf siblings (families

51, 1160, 1548, 1608, 1773, 1873, 1877) and one Australian

(9670) family. Family 1608 was atypical in that four siblings

20   sharing the same DFNB1 marker haplotypes had a mild to

moderate deafness (severe at high frequency), with the child

of one of them being profoundly deaf.   In family 1873, the

unrelated parents (individuals II.2 and II.3) were deaf as

well as their two children, and we have therefore considered

14

this as two families, bringing to nine the total of independent families. Apart from families 1608 and 1873, no parent acknowledged any hearing impairment. These nine families showed cosegregation between deafness and

5   polymorphic markers of the DFNB1 region with maximum individual lod scores ranging from 0.6 to 1.2. Ten other families in the original study of Maw et al. (Maw MA, Allen-Powell DR, Goodey RJ, et al. The contribution of the DFNB1 locus to neurosensory deafness in a Caucasian population. *Am*

10   *J Hum Genet* 1995; 57:629-35) had shown no cosegregation and one other cosegregating family was not tested for Cx26 mutations. The New Zealand families were all of Caucasian origin with no known Polynesian admixture. According to the antecedent family names, the ancestral proportion among the

15   families reflected that of the general Caucasian New Zealand population with the great predominance being of Anglo-Celtic patrimony and a small fraction due to migration from continental Europe. Neither parental consanguinity, nor links between any of the families were recognized. In the

20   Australian case, the father was from Northern Ireland and the mother from Yorkshire, England.

The third category is composed of nineteen families living in France and two in New Zealand, each with at least two children having a severe to profound deafness. No parent

acknowledged any hearing impairment, except for the mother in
family P16 and the father in family P17 who had moderate and
progressive high-frequency hearing loss. Five of these
families had foreign ancestors from Lebanon (family P3),
5   Turkey (family P4), Portugal (family P9), Algeria (family
P14) and Poland (father in family P16). In two of the
families (P7 and P14), the parents were distantly related.

Example 2: Amplification of the coding exon of Cx26
10   PCRs were carried out on genomic DNA using a set of primers
that allowed the amplification of the entire coding sequence
of the Cx26 gene, which consists of a single coding exon
(Kelsell DP, Dunlop J, Stevens HP, et al. Connexin 26
mutations in hereditary non-syndromic sensorineural deafness.
15   *Nature* 1997; **387**: 80-3). Primer sequences were as follows:

      5'-TCTTTTCCAGAGCAAACCGCC(SEQ ID No. 1)-3' and

      5'-TGAGCACGGGTTGCCTCATC(SEQ ID No. 2)-3'.

PCR conditions were: 35 cycles of 95°C, 1 min; 58°C, 1 min;
72°C, 2 min. The PCR product obtained was 777 bp in length.
20

Example 3: DNA sequencing

      Sequencing of the PCR products was performed as
previously described (Smith LM, Sanders JZ, Kaiser RJ, et

al., Fluorescence detection in automated DNA sequence
analysis, *Nature* 1986; **321**:674-9) using the dideoxy chain
terminator method on an Applied Biosystems DNA sequencer
ABI373 with fluorescent dideoxynucleotides. The primers used
5   were the same as those for the PCR amplification plus two
internal primers

     5'-GACACGAAGATCAGCTGCAG(SEQ ID No. 3)-3' and

     5'-CCAGGCTGCAAGAACGTGTG(SEQ ID No. 4)-3'.

10   Example 4: Mutations in consanguineous Tunisian and
     Lebanese DFNB1 families

         In these families the involvement of the DFNB1 locus
     could be demonstrated by linkage analysis. In four of the
     five families from Tunisia (S15, S19, 20, and 60) and in the
15   Lebanese family (LH), the same mutation was detected in all
     affected children on both Cx26 alleles, namely, a deletion of
     a guanosine (G) in a sequence of six G extending from
     position 30 to 35 (position 1 being the first base of the
     initiator codon) (Table 1). This mutation is hereafter
20   referred to as 30delG mutation according to the nomenclature
     proposed by Beaudet and Tsui ((Beaudet AL, Tsui L-C. A
     suggested nomenclature for designating mutations, *Hum
     Mutation* 1993; **2**: 245-8)). It creates a frameshift, which
     results in a premature stop codon at nucleotide position 38.

The mutation segregating in the fifth family from Tunisia (ST) was identified as a G to T transversion at nucleotide position G39 creating a premature stop codon (GAG->TAG) at codon 47, and was designated E47X. In each family, normal

5 hearing parents were found to be heterozygous for the corresponding mutation.

Example 5:   Mutations in small nonconsanguineous New Zealand   and Australian families consistent with DFNB1

10 linkage

In these families, segregation analysis has previously been reported as compatible with the involvement of the DFNB1 locus (Maw MA, Allen-Powell DR, Goodey RJ, et al.   The contribution of the DFNB1 locus to neurosensory deafness in

15 a Caucasian population. *Am J Hum Genet* 1995; **57**: 629-35). The deaf individuals from five of the nine families (51, 1160, 1608 (III.20), 1873 (II.3) and 1877) were homozygous for the 30delG mutation. The deaf children from family 1773 were heterozygous for 30delG.   Deaf individual II.2 from

20 family 1873 (see "subjects" and Table 1) was heterozygous for a deletion of 38 bp beginning at nucleotide position G30, designated 30del38. No other mutation was detected in the deaf children of family 1773 and the deaf individual (II.2) in family 1873. Nevertheless, in this last individual, a

deletion of the polymorphic marker immediately proximal to

the Cx26 gene (locus D13S175) had previously been observed

(Maw MA, Allen-Powell DR, Goodey RJ, et al. The contribution

of the DFNB1 locus to neurosensory deafness in a Caucasian

5   population. *Am J Hum Genet* 1995; **57**: 629-35), which may

indicate   that   a   DNA   rearrangement   has   impaired   the

functioning of the other Cx26 allele of the gene in cis. In

family 9670, compound heterozygosity for a missense mutation

(R184P) and an in frame single-codon deletion (delE138) was

10   observed in affected siblings. In only one family (1548) was

no Cx26 mutation detected. Results are summarized in Table

1.

       Example 6: Mutations in small families uncharacterized

15   for DFNB1 linkage living in France and New Zealand

       Nineteen families (P1 to 17, L14190 and L13131) living

in France and two in New Zealand (families 1885 and 2254)

were studied.   In these families, cosegregation of the

deafness with polymorphic markers had not been analysed. Deaf

20   children from six of the twenty-one families (P1, P3, P5, P9,

P10, and P16) were found to be homozygous for the mutation

30delG. In five additional families (P6, P11, P14, P17, and

1885), deaf children were heterozygous for this mutation; no

other mutation was detected in these families. In the ten

remaining families, no mutation in the Cx26 gene was found.

Example 7:   Molecular hybridization using allele-specific
5   capture probes

Molecular hybridization capture probe (see, e.g., D.

Chevrier et al. PCR product quantification by non-radioactive

hybridization procedures using an oligonucleotide covalently

10   bound to microwells. Molecular and Cellular Probes 1993; 7:

187-197 and D Chevrier et al. Rapid detection of Salmonella

subspecies   I   by   PCR   combined   with   non-radioactive

hybridization using covalently immobilized oligonucleotide on

a microplate. FEMS Immunology and Medical Microbiology 1995;

15   10: 245-252 each of which is incorporated by reference

herein) permit specific detection of the 30delG mutation.

The technique has been adapted to permit rapid diagnosis of

prelingual non-syndromic deafness caused by the 30delG

mutation.   The technique provides certain advantages in a

20   clinical setting because it uses stable, nonradioactive

molecules, it can be easily automated, and it is well adapted

to large scale analysis.

Using primers designed for PCR amplification, the region

of interest in the Cx26 gene is amplified from genomic DNA

25   samples. The primer sequences are as follows:

CONN3: 5'-CTAGTGATTCCTGTGTTGTGTGC(SEQ ID No. 9)-3'

20

CONN4: 5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3'

PCR is performed with the CONN3 (SEQ ID No. 9) and CONN4 (SEQ

ID No. 10) primers (1 μM each), an aliquot of the DNA to be

analyzed (2 μl, 100-300 ng), 1.5 mM $MgCl_2$, 200 μM dNTP, and

5   Taq polymerase. The amplification program consists of the

following steps: 1) 95°C, 5 min; 2) addition of enzyme, 95°C,

1 min; 3) 60°C, 1 min (ramp rate = 0.25°C/s); 4) 72°C, 1 min;

5) repeat steps 2 to 4 for 40 cycles; and 6) 72°C, 10 min.

PCR products are verified by a rapid gel electrophoresis.

10   The amplified PCR product contains either the normal or

the mutant Cx26 sequence. To distinguish between the normal

and mutant sequence, two capture probes are designed. The

sequences of these two capture probes are as follows:

For detection of normal sequence:

15   CONN6: 5'-AAAAAAAATCCTGGGGGGTGTG(SEQ ID No. 11)-3'

For detection of mutant sequence:

CONN7: 5'-AAAAAAAATCCTGGGGGTGTGA(SEQ ID No. 12)-3'

Each capture probe must be 22 nucleotides long. Furthermore,

to be efficient, the capture probe must include an A_7 spacer

20   at its 5' end and a hybridization region of 15 bases. Such

a capture probe is able to specifically differentiate the

mutant sequence from the normal sequence. Thus, CONN6 (SEQ

ID No. 11) is designed to specifically hybridize with the

normal sequence, whereas CONN7 (SEQ ID No. 12) is designed to
specifically hybridize with the mutant sequence.

Before attaching the capture probes to a microtiter
plate, they are phosphorylated at their 5' ends.   The

5   phosphorylation is carried out for 1 hour at 37°C in presence
of 20 nmoles of CONN6 (SEQ ID No. 11) or CONN7 (SEQ ID No.
12) oligonucleotides, 100 µM ATP, 10 units T4 polynucleotide
kinase in 200 µl of buffer (50 mM Tris-HCl pH 7.4; 10 mM
$MgCl_2$; 5mM dithiothreitol; and 1 mM spermidine). The mixture

10   is heated for 10 min. at 68°C to inactivate the T4
polynucleotide kinase, then the oligonucleotide is
precipitated by adding 145 µl of 10 M CH_3COONH_4, 15 µl H2O,
and 800 µl iced ethanol. After a 30 min. incubation in ice,
the mixture is centrifuged for 20 min. at 12,000 x g at 4°C.

15   The resulting pellet is washed with 500 µl iced ethanol
(70%) and dissolved in 800 µl of TE buffer.   The
phosphorylated oligonucleotide concentration is determined by
optical density at 260 nm.

Before attaching the phosphorylated oligonucleotides to

20   microplates, they are denatured by heating at 95°C for 10
min. and rapidly cooled in ice to avoid the formation of
secondary structure. 500 ng of phosphorylated CONN6 (SEQ ID
No. 11) or CONN7 (SEQ ID No. 12) and 1 µl of 1 M 1-

methylimidazole, pH 7, is added to each well of a microplate,

which is kept on ice.   The total volume of each well is

adjusted to 70 µl with distilled water, before adding 30 µl

of a cold, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide

5   solution (167 mM).   The microplate is covered and incubated

for 5 hours at 50°C in an incubator (Thermomix® from

Labsystems).   After the 5-hour incubation, the microplate is

washed three times with a warm solution (50°C) of 0.4 N NaOH

containing 0.25% SDS.   The microplate is incubated for 5 min.

10   with the same warm solution and washed again with warm

NaOH/SDS (50°C).   Finally, the microplate is washed five

times with TE buffer.   The coated microplate can be kept

several months at 4°C, if the wells are filled with TE

buffer.

15   The amplified sequences from the genomic DNA samples are

incubated with a biotinylated detection probe in the coated

microplates.   Unlike the capture probes, which are allele

specific, the detection probe can hybridize with both the

normal and mutant sequences.   The sequence of the detection

20   probe is:

CONN12:   5'-CAGCATTGGAAAGATCTGGCTCA(SEQ ID No. 13)-3'.

The amplified sequences and the detection probe, which is

biotinylated at its 5' end, are denatured directly in the

microplates by successively adding to each well: 95 µl of

23

water, 5 µl of PCR reaction, 40 µl of biotinylated probe (SEQ

ID No. 13) at 22 nM diluted in water, and 14 µl 1 N NaOH.

After 10 min., 21 µl of 1 M NaH_2PO_4 and 1% Sarkosyl is added

to each well to bring the total volume to 175 µl per well.

5   The final concentration of the detection probe is 5 nM. The

microplate is covered and incubated overnight at 40°C in an

incubator (Thermomix® from Labsystems) and then extensively

washed (5 times) with TBS-Tween to remove the excess

biotinylated probe (SEQ ID No. 13).

10   An immunoenzymatic method is used to detect the

hybridized probe. Each well receives 100 µl of the conjugate

(Extravidine - alkaline phosphatase, Sigma E-2636) diluted

1/4000 in TBS-BSA-Tween. The microplate is covered and

incubated for 1 hour at 25°C. Following the incubation, the

15   microplate is washed 5 times with TBS-Tween. Then 200 µl of

preheated (37°C) substrate (7.5 mg para-nitro-phenyl-

phosphate in 20 ml of the following buffer: 1 M

diethanolamine pH 9.8 containing 1mM $MgCl_2$) are added to each

well. The microplate is covered and incubated for 3 hours at

20   37°C. The absorbance is measured at 405 nm to determine the

specific signal and at 630 nm to determine the background

noise.

24

The hybridization ratio (R) between the signal obtained with CONN6 (SEQ ID No. 11) probe (normal sequence) and that obtained with CONN7 (SEQ ID No. 12) probe (mutant sequence) is calculated. The calculated R values are used to determine

5   the genotypes of the sample DNA as follows: homozygous for the normal Cx26 sequence ($R \geq 2$), heterozygous for the 30delG mutation ($0.5 < R < 2$), and homozygous for the 30delG mutation ($R \leq 0.5$). The range of the hybridization ratio (R) can be slightly modified when the number of samples

10   increases. The following table represents an example of results obtained with 39 samples.

	Hybridization ratio (R)		
Genotype :	Normal	Homozygous 30delG	Heterozygous
	5.96	0.48	1.33
	5.43	0.17	1.13
	3.39	0.21	0.73
	4.14	0.16	0.63
	4.09	0.28	1.4
	2.76	0.13	0.73
	2.2	0.21	0.76
	3.97	0.4	0.73
	4.07		1.06
	3		
	2.76		
	3.66		
	3.87		
	3.92		
	3.26		
	5.17		
	2.74		
	4.51		
	6.3		
	3.49		
	4.05		
	3.17		
Number	22	8	9
Mean value	3.91	0.26	0.94
Standard deviation	1.06	0.12	0.29
Range	(6.3 - 2.2)	(0.48 - 0.13)	(1.4 - 0.63)

26

Example 8:   Temperature gradient gel electrophoresis

        Temperature gradient gel electrophoresis (TGGE) permits

the detection of any type of mutation, including deletions,

5   insertions, and substitutions, which is within a desired

region of a gene.   (See, e.g. D. Reiner et al. Temperature-

gradient gel electrophoresis of nucleic acids: Analysis of

conformational transitions, sequence variations and protein-

nucleic acid interactions. Electrophoresis 1989; 10: 377-389;

10 E.P. Lessa and G. Applebaum Screening techniques for

detecting allelic variation in DNA sequences. Molecular

Ecology 1993; 2: 119-129 and A.L. Börresen-Dale et al.

Temporal Temperature Gradient Gel Electrophoresis on the D

code™ System. Bio-Rad US/EG Bulletin 2133; the entire

15 disclosure of each publication is incorporated by reference

herein.)   However, TGGE does not permit one to determine

precisely the type of mutation and its location.

        As in the previously described molecular hybridization

technique, the region of interest in the Cx26 gene is first

20 amplified from genomic DNA samples by PCR.   The primer

sequences are as follows:

        CONN2:   5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCT

                    AGTGATTCCTGTGTTGTGTGC(SEQ ID No. 14)-3'

        CONN4:   5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3'

PCR is performed with 1 µM of the CONN2 (SEQ ID No. 14) primer, which has a GC clamp at its 5' end, and 1 µM of the CONN4 (SEQ ID No. 10) primer, an aliquot of the DNA to be analyzed (2 µl, 100-300ng), 1.5 mM MgCl$_2$, 200 µM dNTP, and

5   Taq polymerase. The amplification program consists of the following steps: 1) 95°C, 5 min; 2) addition of enzyme, 95°C, 1 min; 3) 60°C, 1 min (ramp rate = 0.25°C/s); 4) 72°C, 1 min; 5) repeat steps 2 to 4 for 40 cycles; and 6) 72°C, 10 min.

Analyzing these PCR amplification fragments by TGGE can

10 differentiate between homozygous (normal or mutant) samples, which produce a single band on a gel, and heterozygous samples, which produce three bands. However, differentiating between genomic samples that are homozygous for the normal sequence and genomic samples that are homozygous for the

15 30delG mutants requires an additional step.

To differentiate normal homozygous versus mutant homozygous samples, an aliquot of the amplified PCR product is mixed with either a known, normal homozygous sample or a known, 30delG mutant homozygous sample and analyzed for

20 heteroduplex formation. If the amplified PCR product derives from a normal, homozygous sample, it will form a heteroduplex with the known, 30delG mutant homozygous sample. On the other hand, if the amplified PCR product derives from a

mutant, homozygous sample, it will form a heteroduplex with the known, normal homozygous sample. To promote heteroduplex formation in these mixtures, they are denatured at 95°C for 5 min, followed by a renaturation step at 60°C for 45 min.

5   The PCR fragments from the initial amplification and those that are subjected to the additional heating steps to permit heteroduplex formation are analyzed on a 10% polyacrylamide gel containing 7 M urea. By way of example, a 30 ml gel is prepared by combining the following

10   ingredients:

- 12.6 g urea

- 0.75 ml 50X TAE

- 7.5 ml acrylamide:bisacrylamide (37.5:1) at 40%

- water to bring volume to 30 ml

15   - 30 µl Temed (added extemporaneously)

- 300 µl 10% ammonium persulfate (added extemporaneously).

After adding the Temed and ammonium persulfate, the gel is poured between two glass plates (Dcode Universal Mutation

20   Detection System® from BIORAD) and allowed to polymerize for 1 hour.

An aliquot (7.5 µl) of the PCR mixture is mixed with 7.5 µl of 2X sample solution (2 mM EDTA pH 8; 70% glycerol;

0.05% xylene cyanol; 0.05% bromophenol blue), and introduced
into a gel well. Electrophoresis is performed for 4-5 hours
at 150V in 1.25X TAE buffer with a temperature gradient
ranging from 61°C to 62°C at a rate of 0.2°C per hour.

5   Following electrophoresis, the gel is incubated for 6 min. in
1.25X TAE containing 25 µg/ml ethidium bromide.   Excess
ethidium bromide is removed by a 20 min. wash in 1.25X TAE,
and   the   DNA   fragments   are   visualized   with   a   UV
transilluminator.

10   A typical TGGE result is represented in Figure 1.   The.
amplified DNA from homozygous patients (normal or mutant)
produces only one band.   The amplified DNA from heterozygous
patients   results   in   three   different   fragments   in   the
polyacrylamide gel.   The more intense band, which migrates

15   more rapidly, corresponds to both homoduplexes, which cannot
be separated in this gel.   The other two bands, which migrate
more slowly, correspond to both kinds of heteroduplexes.

         The   DNA   of   normal   homozygous   patients   can   be
differentiated from the DNA of mutant homozygous patients by

20   analyzing   the   PCR   fragments   that   were   subjected   to   the
conditions   that   permitted   heteroduplex   formation.
Heteroduplexes form when the PCR amplified fragment from a
normal homozygous genome is mixed with sequences from a
known, mutant homozygous genome, or when the PCR amplified

fragment from a mutant homozygous genome is mixed with
sequences from a known, normal homozygous genome. These
heteroduplexes are visible by TGGE analysis. Consequently,
the DNA of normal and mutant homozygous patients can be
5   easily differentiated by this technique using the primers
described in the present study.

                              *   *   *

     In all the known DFNB1 families (6/6), in all but one
(8/9) of the putatively DFNB1-linked families, and in about
10   half (11/21) of the families not tested for DFNB1 linkage, a
mutation in Cx26 was detected.   Furthermore, of the 44
chromosomes reckoned to be independent upon which a Cx26
mutant allele was identified or inferred, 33(75%) were found
to carry the same deletion of a guanosine, G, at position 30
15   (30delG).

     Cx26 mutations represent a major cause of recessively
inherited prelingual deafness and would be implicated in
about half of cases in the examined populations.   In
addition, one specific mutation, 30delG, accounts for the
20   majority (about three-quarters in our series) of the Cx26
mutant alleles.

     The wild type connexin 26 gene published in LEE S.W. et
al. (1992)J. Cell Biol. 118: 1213-1221 has the following
sequence:

```
   1 GATTTAATCC TATGACAAAC TAAGTTGGTT CTGTCTTCAC CTGTTTTGGT
  51 GAGGTTGTGT AAGAGTTGGT GTTTGCTCAG GAAGAGATTT AAGCATGCTT
 101 GCTTACCCAG ACTCAGAGAA GTCTCCCTGT TCTGTCCTAG CTATGTTCCT
 151 GTGTTGTGTG CATTCGTCTT TTCCAGAGCA AACCGCCCAG AGTAGAAGAT
 201 GGATTGGGGC ACGCTGCAGA CGATCCTGGG GGGTGTGAAC AAACACTCCA
 251 CCAGCATTGG AAAGATCTGG CTCACCGTCC TCTTCATTTT TCGCATTATG
 301 ATCCTCGTTG TGGCTGCAAA GGAGGTGTGG GGAGATGAGC AGGCCGACTT
 351 TGTCTGCAAC ACCCTGCAGC CAGGCTGCAA GAACGTGTGC TACGATCACT
 401 ACTTCCCCAT CTCCCACATC CGGCTATGGG CCCTGCAGCT GATCTTCGTG
 451 TCCAGCCCAG CGCTCCTAGT GGCCATGCAC GTGGCCTACC GGAGACATGA
 501 GAAGAAGAGG AAGTTCATCA AGGGGGAGAT AAAGAGTGAA TTTAAGGACA
 551 TCGAGGAGAT CAAAACCCAG AAGGTCCGCA TCGAAGGCTC CCTGTGGTGG
 601 ACCTACACAA GCAGCATCTT CTTCCGGGTC ATCTTCGAAG CCGCCTTCAT
 651 GTACGTCTTC TATGTCATGT ACGACGGCTT CTCCATGCAG CGGCTGGTGA
 701 AGTGCAACGC CTGGCCTTGT CCCAACACTG TGGACTGCTT TGTGTCCCGG
 751 CCCACGGAGA AGACTGTCTT TCACAGTGTT CATGATTGCA GTGTCTGGAA
 801 TTTGCATCCT GCTGAATGTC ACTGAATTGT GTTATTTGCT AATTAGATAT
 851 TGTTCTGGGA AGTCAAAAAA GCCAGTTTAA CGCATTGCCC AGTTGTTAGA
 901 TTAAGAAATA GACAGCATGA GAGGGATGAG GCAACCCGTG CTCAGCTGTC

 951 AAGGCTCAGT CGCCAGCATT TCCCAACACA AAGATTCTGA CCTTAAATGC
1001 AACCATTTGA AACCCCTGTA GGCCTCAGGT GAAACTCCAG ATGCCACAAT
1051 GAGCTCTGCT CCCCTAAAGC CTCAAAACAA AGGCCTAATT CTATGCCTGT
1101 CTTAATTTTC TTTCACTTAA GTTAGTTCCA CTGAGACCCC AGGCTGTTAG
1151 GGGTTATTGG TGTAAGGTAC TTTCATATTT TAAACAGAGG ATATCGGCAT
1201 TTGTTTCTTT CTCTGAGGAC AAGAGAAAAA AGCCAGGTTC CACAGAGGAC
1251 ACAGAGAAGG TTTGGGTGTC CTCCTGGGGT TCTTTTTGCC AACTTTCCCC
1301 ACGTTAAAGG TGAACATTGG TTCTTTCATT TGCTTTGGAA GTTTTAATCT
1351 CTAACAGTGG ACAAAGTTAC CAGTGCCTTA AACTCTGTTA CACTTTTTGG
1401 AAGTGAAAAC TTTGTAGTAT GATAGGTTAT TTTGATGTAA AGATGTTCTG
1451 GATACCATTA TATGTTCCCC CTGTTTCAGA GGCTCAGATT GTAATATGTA
```

```
1501  AATGGTATGT  CATTCGCTAC  TATGATTTAA  TTTGAAATAT  GGTCTTTTGG
1551  TTATGAATAC  TTTGCAGCAC  AGCTGAGAGA  GGCTGTCTGT  TGTATTCATT
1601  GTGGTCATAG  CACCTAACAA  CATTGTAGCC  TCAATCGAGT  GAGACAGACT
1651  AGAAGTTCCT  AGTTGGCTTA  TGATAGCAAA  TGGCCTCATG  TCAAATATTA
1701  GATGTAATTT  TGTGTAAGAA  ATACAGACTG  GATGTACCAC  CAACTACTAC
1751  CTGTAATGAC  AGGCCTGTCC  AACACATCTC  CCTTTTCCAT  GCTGTGGTAG
1801  CCAGCATCGG  AAAGAACGCT  GATTTAAAGA  GGTGAGCTTG  GGAATTTTAT
1851  TGACACAGTA  CCATTTAATG  GGGAGACAAA  AATGGGGGCC  AGGGGAGGGA
1901  GAAGTTTCTG  TCGTTAAAAA  CGAGTTTGGA  AAGACTGGAC  TCTAAATTCT
1951  GTTGATTAAA  GATGAGCTTT  GTCTACCTTC  AAAAGTTTGT  TTGGCTTACC
2001  CCCTTCAGCC  TCCAATTTTT  TAAGTGAAAA  TATAACTAAT  AACATGTGAA
2051  AAGAATAGAA  GCTAAGGTTT  AGATAAATAT  TGAGCAGATC  TATAGGAAGA
2101  TTGAACCTGA  ATATTGCCAT  TATGCTTGAC  ATGGTTTCCA  AAAAATGGTA
2151  CTCCACATAG  TTCAGTGAGG  GTAAGTATTT  TCCTGTTGTC  AAGAATAGCA
2201  TTGTAAAAGC  ATTTTGTAAT  AATAAAGAAT  AGCTTTAATG  ATATGCTTGT
2251  AACTAAAATA  ATTTTGTAAT  GTATCAAATA  CATTTAAAAC  ATTAAAATAT
2301  AATCTCTATA  AT
```

5   The wild type connexin 26 gene published in Kiang, D.T.

et al. (1997) Gene 199 (1-2): 165-171; has the following

sequence:

```
      1  GATTAATCC  TATGACAAAC  TAAGTTGGTT  CTGTCTTCAC  CTGTTTTGGT
10   51  GAGGTTGTGT  AAGAGTTGGT  GTTTGCTCAG  GAAGAGATTT  AAGCATGCTT
     101  GCTTACCCAG  ACTCAGAGAA  GTCTCCCTGT  TCTGTCCTAG  CTAGTGATTC
     151  CTGTGTTGTG  TGCATTCGTC  TTTTCCAGAG  CAAACCGCCC  AGAGTAGAAG
15
     201  ATGGATTGGG  GCACGCTGCA  GACGATCCTG  GGGGGTGTGA  ACAAACACTC
```

```
 251   CACCAGCATT GGAAAGATCT GGCTCACCGT CCTCTTCATT TTTCGCATTA

 301   TGATCCTCGT TGTGGCTGCA AAGGAGGTGT GGGGAGATGA GCAGGCCGAC

 351   TTTGTCTGCA ACACCCTGCA GCCAGGCTGC AAGAACGTGT GCTACGATCA

 401   CTACTTCCCC ATCTCCACA TCCGGCTATG GGCCCTGCAG CTGATCTTCG

 451   TGTCCACGCC AGCGCTCCTA GTGGCCATGC ACGTGGCCTA CCGGAGACAT

 501   GAGAAGAAGA GGAAGTTCAT CAAGGGGGAG ATAAAGAGTG AATTTAAGGA

 551   CATCGAGGAG ATCAAAACCC AGAAGGTCCG CATCGAAGGC TCCCTGTGGT

 601   GGACCTACAC AAGCAGCATC TTCTTCCGGG TCATCTTCGA AGCCGCCTTC

 651   ATGTACGTCT CTATGTCAT GTACGACGGC TTCTCCATGC AGCGGCTGGT

 701   GAAGTGCAAC GCCTGGCCTT GTCCCAACAC TGTGGACTGC TTTGTGTCCC

 751   GGCCCACGGA GAAGACTGTC TTTCACAGTG TTCATGATTG CAGTGTCTGG

 801   AATTTGCATC CTGCTGAATG TCACTGAATT GTGTTATTTG CTAATTAGAT

 851   ATTGTTCTGG GAAGTCAAAA AAGCCAGTTT AACGCATTGC CCAGTTGTTA

 901   GATTAAGAAA TAGACAGCAT GAGAGGGATG AGGCAACCCG TGCTCAGCTG

 951   TCAAGGCTCA GTCGCCAGCA TTTCCCAACA CAAAGATTCT GACCTTAAAT

1001   GCAACCATTT GAAACCCCTG TAGGCCTCAG GTGAAACTCC AGATGCCACA

1051   ATGAGCTCTG CTCCCCTAAA GCCTCAAAAC AAAGGCCTAA TTCTATGCCT

1101   GTCTTAATTT TCTTTCACTT AAGTTAGTTC CACTGAGACC CCAGGCTGTT

1151   AGGGGTTATT GGTGTAAGGT ACTTTCATAT TTTAAACAGA GGATATCGGC

1201   ATTTGTTTCT TTCTCTGAGG ACAAGAGAAA AAAGCCAGGT TCCACAGAGG

1251   ACACAGAGAA GGTTTGGGTG TCCTCCTGGG GTTCTTTTTG CCAACTTTCC

1301   CCACGTTAAA GGTGAACATT GGTTCTTTCA TTTGCTTTGG AAGTTTTAAT

1351   CTCTAACAGT GGACAAAGTT ACCAGTGCCT TAAACTCTGT TACACTTTTT

1401   GGAAGTGAAA ACTTTGTAGT ATGATAGGTT ATTTTGATGT AAAGATGTTC
```

SUBSTITUTE SHEET (RULE 26)

```
   1451  TGGATACCAT TATATGTTCC CCCTGTTTCA GAGGCTCAGA TTGTAATATG

   1501  TAAATGGTAT GTCATTCGCT ACTATGATTT AATTTGAAAT ATGGTCTTTT

 5 1551  GGTTATGAAT ACTTTGCAGC ACAGCTGAGA GAGGCTGTCT GTTGTATTCA

   1601  TTGTGGTCAT AGCACCTAAC AACATTGTAG CCTCAATCGA GTGAGACAGA

   1651  CTAGAAGTTC CTAGTTGGCT TATGATAGCA AATGGCCTCA TGTCAAATAT
10
   1701  TAGATGTAAT TTTGTGTAAG AAATACAGAC TGGATGTACC ACCAACTACT

   1751  ACCTGTAATG ACAGGCCTGT CCAACACATC TCCCTTTTCC ATGCTGTGGT

15 1801  AGCCAGCATC GGAAAGAACG CTGATTTAAA GAGGTGAGCT TGGGAATTTT

   1851  ATTGACACAG TACCATTTAA TGGGGAGACA AAAATGGGGG CCAGGGGAGG

   1901  GAGAAGTTTC TGTCGTTAAA AACGAGTTTG GAAAGACTGG ACTCTAAATT
20
   1951  CTGTTGATTA AAGATGAGCT TTGTCTACCT TCAAAGTTT GTTTGGCTTA

   2001  CCCCCTTCAG CCTCCAATTT TTTAAGTGAA AATATAACTA ATAACATGTG

25 2051  AAAAGAATAG AAGCTAAGGT TTAGATAAAT ATTGAGCAGA TCTATAGGAA

   2101  GATTGAACCT GAATATTGCC ATTATGCTTG ACATGGTTTC CAAAAAATGG

   2151  TACTCCACAT ACTTCAGTGA GGGTAAGTAT TTTCCTGTTG TCAAGAATAG
30
   2201  CATTGTAAAA GCATTTGTA ATAATAAAGA ATAGCTTTAA TGATATGCTT

   2251  GTAACTAAAA TAATTTTGTA ATGTATCAAA TACATTTAAA ACATTAAAAT

35 2301  ATAATCTCTA TAAT
```

(SEQ ID No. 8). The ATG underlined in the sequences corresponds to the start codon. The guanine residue "G", which is in bold print, marks the end of the guanosine rich region between nucleotides 27 and 32, inclusive.

35

Table 1:   Mutations in the Cx26 coding exon in individuals
           affected   with   familial   forms   of   prelingual
           deafness

Family (geographical origin)	30delG mutation	Other mutation	Deafness
DFNB1-linked families			
S15 (sTu)	homozygous	-	profound
S19 (sTu)	homozygous	-	profound
ST (sTu)	-	homozygous E47X	profound
20 (nTu)	homozygous	-	profound
60 (nTu)	homozygous	-	profound
LH (Leb)	homozygous	-	severe-profound
Families consistent with DFNB1 linkage			

51 (NZ)	homozygous	-	severe-profound
1160 (NZ)	homozygous	-	moderate-severe*
1548 (NZ)	-	-	profound
1608 (NZ)	homozygous	-	profound**
1773 (NZ)	heterozygous	-	profound
1873 individual II.3 (NZ)	homozygous	-	moderate
1873 individual II.2 (NZ)	-	heterozygous 30del38	profound
1877 (NZ)	homozygous	-	profound
9670 (Aust)		delE118/R148P	moderate-severe
Families uncharacterized for DFNB1 linkage			
P1 (Fr)	homozygous	-	severe-profound
P2	-	-	profound

(Fr)			
P3	homozygous	-	severe-profound
(Leb)			
P4	-	-	severe-profound
(Tur)			
P5	homozygous	-	profound
(Fr)			
P6	heterozygous	-	severe-profound
(Fr)			
P7	-	-	moderate
(Fr)			
P8	-	-	moderate
(Fr)			
L13131 (Fr)	-	-	profound
L14190 (Fr)	-	-	mild-moderate
P9	homozygous	-	severe-profound
(Por)			
P10	homozygous	-	severe-profound
(Fr)			
P11	heterozygous	-	moderate-severe
(Fr)			
P12	-	-	severe-profound
(Fr)			
P13	-	-	profound
(Fr)			

38

P14 (Alg)	heterozygous	-	moderate-severe
P15 (Fr)	-	-	severe-profound
P16 (mother/Fr, father/Pol)	homozygous	-	severe**
P17 (Fr)	heterozygous	-	severe***
1885 (NZ)	heterozygous	-	profound
2254 (NZ)	-	-	moderate-severe

The analysis reported here concerns deaf children of the various families except for family 1873 (see patients and methods).

5 * moderate in one ear, severe in the other ear.

**moderate hearing loss in mother (severe at high frequencies), ***mild hearing loss in father, who are heterozygous carriers for the 30delG mutation.

Geographical origins: (Alg) Algeria, (Aust) Australia, (Fr)

10 France, (Leb) Lebanon, (NZ) New Zealand, (Pol) Poland, (Por) Portugal, (nTu) North Tunisia, (sTu) South Tunisia, (Tur) Turkey

What is claimed is:

1. A purified polynucleotide having a chain of
5   nucleotides corresponding to a mutated sequence, which in a
wild form encodes a polypeptide implicated in hereditary
sensory defect, wherein said mutated purified polynucleotide
presents a mutation responsible for prelingual non-syndromic
deafness selected from the group consisting of a specific
10 deletion of at least one nucleotide.

2. The purified polynucleotide according to claim 1,
wherein the specific deletion is located in a region encoding
connexin 26 of chromosome 13q11-12.

3. The purified polynucleotide according to claim 1 or
15 2, wherein the specific deletion is located in a guanosine
rich region starting at nucleotide 27, preferably at
nucleotide 30 and extending to nucleotide 32 or nucleotide
35, (position 1 being the first base of the initiator codon).

4. The purified polynucleotide according to any one of
20 claims 1 to 3, wherein the specific deleted polynucleotide
encodes a truncated polypeptide.

5. The purified polynucleotide according to any one of
claims 1 to 3, wherein the specific deletion is a guanosine
deletion at position 30.

25   6. The purified polynucleotide according to any one of
claims 1 to 3, wherein specific deletion is a 38bp deletion
beginning at position 30.

7. A purified polynucleotide, which hybridizes under
stringent conditions specifically with a polynucleotide
30 according to any one of claims 1 to 6.

8. An oligonucleotide useful as a primer or as a probe consisting of 15 to 50 consecutive nucleotides of the polynucleotide according to claim 7.

9. A pair of oligonucleotides according to claim 8,
5   consisting of the following sequences:

      5'-TCTTTTCCAGAGCAAACCGCC(SEQ ID No. 1)-3'; and

      5'-TGAGCACGGGTTGCCTCATC(SEQ ID No. 2)-3'.

10. The oligonucleotide according to claim 8, which has a sequence selected from the group consisting of:

10   5'-AGACGATCCTGGGGGTGTGAACAAA(SEQ ID No. 5)-3';

      5'-ATCCTGGGGGTGTGA(SEQ ID No. 6)-3'; and

      5'-AGACGATCCTGGGGGCTCACCGTCCTC(SEQ ID No. 7)-3'.

11. A pair of the oligonucleotides as claimed in claim 8, which are:

15   5'-CTAGTGATTCCTGTGTTGTGTGC(SEQ ID No. 9)-3'; and

      5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3'.

12. A pair of oligonucleotides as claimed in claim 8, which are:

      5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCTAGTGATTCCT
20   GTGTTGTGTGC(SEQ ID No. 14)-3'; and

      5' ATAATGCGAAAAATGAAGAGGA(SEQ ID No. 10)-3'.

13. A method for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous and heterozygous individuals in a biological
25   sample containing DNA, comprising the steps of:

      a) bringing the biological sample into contact with oligonucleotide primers according to any one of claims 8 to 12, the DNA contained in the sample having been optionally made available to hybridization and under conditions
30   permitting a hybridization of the primers with the DNA contained in the biological sample;

      b) amplifying the DNA;

41

c) revealing the amplification products; and

d) detecting the mutation by appropriate techniques.

14. The method of claim 13, wherein in step d) the mutation is detected by one of the following techniques:

- single-strand conformation polymorphism (SSCP); or

- denaturing gradient gel electrophoresis (DGGE); or

- sequencing or

- temperature gradient gel electrophoresis (TGGE).

15. The method according to claim 13 or 14, wherein step c) comprises the detection of the amplified products with an oligonucleotide probe according to any one of claims 8, 9, or 10.

16. A method for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous and heterozygous individuals in a biological sample containing DNA, comprising the steps of:

a) bringing the biological sample into contact with an oligonucleotide probe according to any one of claims 8 to 12, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the biological sample; and

b) detecting the hybrid formed between the oligonucleotide probe and the DNA contained in the biological sample.

17. The method according to any one of claims 13 to 16, wherein before step a), the DNA contained in the biological sample is amplified using a pair of primers.

18. The method of any one of claims 13 to 15, wherein step d) further comprises the steps of:

a) incubating the amplification products with a labeled detection probe that hybridizes with both a normal Cx26

sequence and a 30delG mutant sequence and a first capture probe that hybridizes with said normal Cx26 sequence but does not hybridize with said 30delG mutant sequence;

5   b)   incubating the amplification products with said labeled detection probe and a second capture probe that hybridizes with said mutant 30delG sequence but does not hybridize with said normal Cx26 sequence;

        c)   hybridizing the amplification products with said
10   detection probe and with said first or second capture probe; and

        d)   comparing the hybridization signal obtained from said first capture probe with the hybridization signal obtained from said second capture probe.

15   19.   The method of claim 18, wherein in step a) the biological sample is brought into contact with the pair of oligonucleotide primers as claimed in claim 12.

        20.   The method of claim 18 or 19, wherein said first capture probe is 5'-AAAAAAAATCCTGGGGGGTGTG(SEQ ID No. 11)-3'
20   and   said   second   capture   probe   is   5'-AAAAAAAATCCTGGGGGTGTGA(SEQ ID No. 12)-3'.

        21.   The method of any one of claims 18 to 20, wherein said detection probe is 5-CAGCATTGGAAAGATCTGGCTCA(SEQ ID No. 13)-3'.

25   22.   The method of any one of claims 18 to 21, wherein said detection probe is non-radioactively labeled.

        23.   The method of claim 22, wherein said detection probe is labeled with biotin.

        24.   The method of any one of claims 18 to 23, wherein
30   said first and second capture probes are bound to a microplate.

25.   The method of claim 13, wherein in step a) the biological sample is brought into contact with the pair of oligonucleotide primers according to claim 12.

26.   The method of claim 25 further comprising between
5   step b) and step c):

incubating the amplified DNA with either a first nucleotide sequence from a known, normal homozygous Cx26 sample or a second nucleotide sequence from a known mutant Cx26 sample; and

10   hybridizing the amplified DNA with either said first nucleotide sequence or said second nucleotide sequence.

27.   The method of claim 25 or 26, wherein step d) comprises analyzing the hybridized DNA to differentiate
15   between DNA from normal homozygous Cx26 individuals and DNA from mutant homozygous Cx26 individuals.

28.   A kit for the detection of an hereditary sensory defect, the autosomal prelingual non-syndromic deafness, for homozygous and heterozygous individuals, said kit comprising:

20   a) oligonucleotides as claimed in any one of claims 8, 9, 10, 11 or 12;

b)   reagents   necessary   for   carrying   out   a   DNA amplification; and

c) a component that makes it possible to determine the
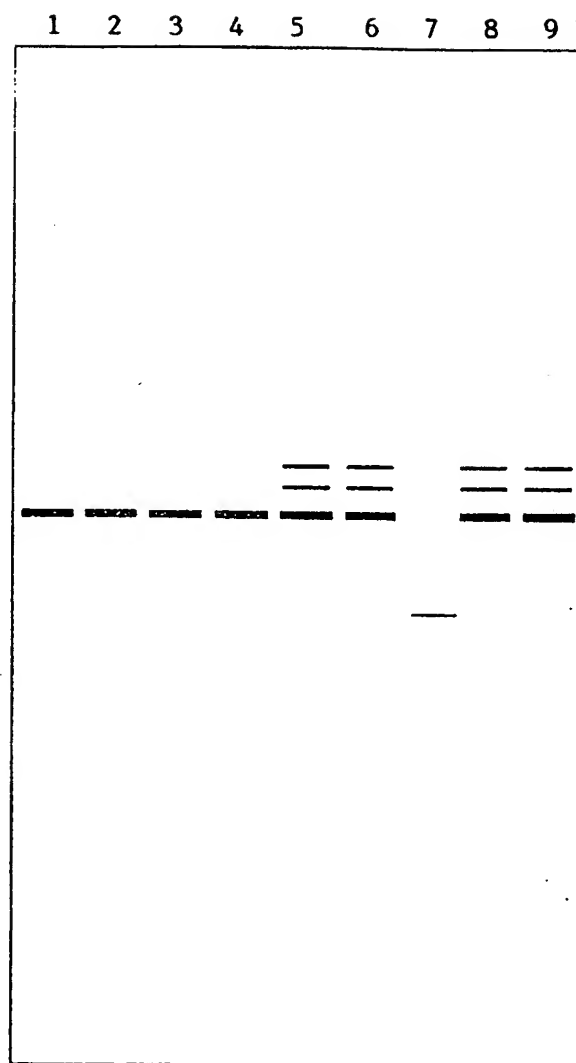25   length of the amplified fragments or to detect a mutation.

Figure 1